Available online at www.sciencedirect.com
ScienceDirect
Veterinary Microbiology 131 (2008) 103–114
veterinary microbiology
www.elsevier.com/locate/vetmic

Effect of porcine circovirus type 2 (PCV2) vaccination on porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2 coinfection

T. Opriessnig [a,*], D.M. Madson [a], J.R. Prickett [a], D. Kuhar [b], J.K. Lunney [b], J. Elsener [c], P.G. Halbur [a]

[a] *Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, 1600 S 16th Street, Ames, IA 50011, USA*
[b] *Animal Parasitic Diseases Laboratory ANRI, ARS, USDA Building 1040, Room 103, BARC-East Beltsville, MD 20705, USA*
[c] *Fort Dodge Animal Health, Inc., 800 5th Street NW, Fort Dodge, IA 50501, USA*

Received 18 December 2007; received in revised form 3 March 2008; accepted 6 March 2008

Abstract

The objectives were to determine if PCV2 vaccination is effective in reducing disease and lesions associated with PRRSV and PCV2 coinfection and if there is a difference between intradermal (ID) and intramuscular (IM) route of PCV2 vaccination. Seventy-four, 21-day-old pigs were randomly allocated into one of six groups. On day 0, pigs were vaccinated with 2 ml Suvaxyn® PCV2 One Dose (Fort Dodge Animal Health, Inc.) by intramuscular (VAC-M-COINF) or intradermal (VAC-D-COINF) routes. On day 28, pigs were either singularly (PRRSV-only, PCV2-only) or coinfected (COINF) with PRRSV and PCV2. All pigs in all groups were necropsied on day 42. All vaccinated pigs seroconverted (IgM, IgG, and neutralizing antibodies) to PCV2 between 14 and 28 days post-vaccination. After challenge, all groups inoculated with PRRSV had reduced average daily gain compared to CONTROLS and PCV2-only ($P < 0.001$). COINF pigs had significantly ($P < 0.05$) reduced anti-PCV2-IgG antibody levels and neutralizing antibody levels compared to both vaccinated groups. COINF pigs had more severe lung lesions compared to VAC-M-COINF ($P < 0.05$). COINF pigs had higher amounts of PCV2 DNA in serum samples and feces ($P < 0.05$) and increased amounts of PCV2 in lymphoid tissues ($P < 0.05$) compared to both vaccinated groups. In summary, PCV2 vaccination was effective at inducing a neutralizing antibody response and significantly reducing PCV2-associated lesions and PCV2 viremia in pigs coinfected with PCV2 and PRRSV. Differences between intradermal and intramuscular routes of vaccine administration were not observed.

Keywords: Porcine circovirus type 2; Porcine reproductive and respiratory syndrome virus; Porcine respiratory disease complex; Vaccination

* Corresponding author. Tel.: +1 515 294 1137; fax: +1 515 294 3564.
E-mail address: tanjaopr@iastate.edu (T. Opriessnig).

0378-1135/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2008.03.002

1. Introduction

Porcine circovirus type 2 (PCV2)-associated disease (PCVAD) was first described in Canada in 1991 (Harding and Clark, 1997) and recently emerged as a major problem in North America. The most common manifestations of PCVAD observed in case submissions are severe systemic and respiratory disease. In both of these manifestations, porcine reproductive and respiratory syndrome virus (PRRSV) is commonly present along with PCV2 (Harms et al., 2002; Dorr et al., 2007). PRRSV has been shown to enhance disease in pigs experimentally coinfected with PCV2 and PRRSV. Allan et al. (2000a) inoculated 1–2-day-old colostrum-deprived pigs with PCV2 and PRRSV and observed upregulation of PCV2 replication in coinfected pigs. However, the replication and distribution of PRRSV in concurrently infected pigs was not enhanced compared to that observed in single PRRSV infected pigs (Allan et al., 2000a). Harms et al. (2001) coinfected 3-week-old cesarian-derived, colostrum-deprived (CDCD) pigs at 3 weeks of age with PCV2 and PRRSV and showed that PCV2 infection increased the severity of PRRSV-induced interstitial pneumonia in CDCD pigs. Rovira et al. (2002) inoculated 5-week-old conventional pigs with PRRSV and 7 days later with PCV2 and confirmed that PRRSV infection enhances PCV2 replication.

Minimizing the effect of PCV2 infection has been an effective strategy for reducing the severity of porcine respiratory disease complex (PRDC). Recently, commercial PCV2 vaccines became available in North America and appear to be effective in reducing losses attributed to PCVAD (Opriessnig et al., 2007). PRRSV and PCV2 are widespread in the pig population and as such it is likely that most herds where PCV2 vaccines are being used are infected with PRRSV yet limited information is available on the efficacy of PCV2 vaccines in pigs experimentally coinfected with PCV2 and PRRSV. The objectives of this study were (1) to determine if PCV2 vaccination is effective in reducing disease and lesions associated with PRRSV and PCV2 coinfection and (2) to determine if there is a difference between intradermal (ID) and intramuscular (IM) route of PCV2 vaccination.

2. Materials and methods

2.1. Animals, housing, and experimental design

Seventy-four, 28-day-old, colostrum-fed, crossbred, specific-pathogen-free (SPF) pigs were purchased from a herd that is routinely tested for major swine pathogens and known to be free of PCV2, PRRSV, and swine influenza virus (SIV). The pigs were weaned at 3 weeks of age and transported to the Livestock Infectious Disease Isolation Facility at Iowa State University, Ames, IA. On the day of arrival, pigs were randomly assigned to one of six groups and rooms. Within each room, 6–7 pigs were contained in 2.5 m × 3.6 m raised wire decks equipped with one nipple drinker and one self-feeder. All groups were fed ad libitum a balanced, pelleted, feed ration with carbadox included at a rate of 50 g/ton. The experimental design is summarized in Table 1. At the start of the experiment (day 0), the pigs were 28 days old. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee.

2.2. Vaccination

On day 0, VAC-M-COINF ($n = 12$) and VAC-D-COINF ($n = 12$) received 2 ml of a commercially available killed PCV1-2 chimeric vaccine (Suvaxyn® PCV2 One Dose™; Fort Dodge Animal Health, Inc., Fort Dodge, IA, USA; Lot number 1861100A). The injection was given intramuscularly (VAC-M-COINF) into the right neck using a sterile 18 gauge 1.5 in. hypodermic needle attached to a 3 ml disposable syringe according to the manufacturer's instructions or intradermally (VAC-D-COINF) by using the Pulse 250™ needleless injection system (Pulse NeedleFree Systems Inc., Lenexa, KS).

2.3. Inoculation

On day 28, PCV2-single, COINF, VAC-M-COINF, and VAC-D-COINF groups were inoculated intranasally (1.5 ml) and intramuscularly (1 ml) with PCV2 40895 (Fenaux et al., 2000, 2002) at a dose of $10^{5.5}$ $TCID_{50}$. On day 28, PRRSV-single, COINF, VAC-M-COINF, and VAC-D-COINF groups were inoculated intranasally with 4 ml PRRSV ISU12 passage 7 at a dose of approximately 10^5 $TCID_{50}$.

Table 1
Summary of the study design including group designation, number of pigs per group (*n*), vaccination status, PCV2 and PRRSV challenge status

Group	*n*	Day 0 vaccination[a]		Day 28 challenge	
		Status	Route	PCV2	PRRSV
CONTROLS	12	Non-vaccinated	–	–	–
PCV2-only	12	Non-vaccinated	–	+	–
PRRSV-only	12	Non-vaccinated	–	–	+
COINF	14	Non-vaccinated	–	+	+
VAC-M-COINF	12	Vaccinated	Intramuscularly	+	+
VAC-D-COINF	12	Vaccinated	Intradermal	+	+

[a] Suvaxyn® PCV2 One Dose™; Fort Dodge Animal Health, Inc., Fort Dodge, IA, USA.

2.4. Serology

Blood samples were collected from all pigs on arrival to the research facility and weekly thereafter until necropsy.

Anti-PCV2-IgM-antibodies: The samples were tested by Ingezim Circovirus IgM ELISA (Ingenasa, Madrid, Spain). The plate-specific ELISA cut-off value was determined by multiplying the average optical density at 450 nm of the IgM positive control wells by 0.4.
Anti-PCV2-IgG-antibodies: The serum samples were tested by a ORF2-PCV2-IgG ELISA as previously described (Nawagitgul et al., 2002). Samples were considered positive if the calculated sample-to-positive (S/P) ratio was 0.2 or greater.
Neutralizing PCV2 antibodies: A fluorescence focus neutralization assay was done to determine the presence and level of neutralizing antibodies against PCV2 as previously described (Pogranichnyy et al., 2000) on day 0, 28, and 42 serum samples from PCV2-only, COINF, VAC-M-COINF, and VAC-D-COINF groups. PCV2 isolate ISU-98-15237 was used in this assay.
Anti-PRRSV-IgG-antibodies: The serum samples taken on days 0, 28, and 42 were tested for the presence of antibodies to PRRSV by a commercial PRRSV ELISA (HerdChek PRRS virus antibody test kit 2XR, IDEXX Laboratories, Inc. Westbrook, MA, USA).

2.5. Clinical evaluation

Following PCV2 inoculation, the pigs were monitored daily for respiratory disease (dyspnea, sneezing, coughing, nasal discharge). Rectal temperatures and behavioral changes such as lethargy were also recorded daily. The pigs were weighed on the day of vaccination and weekly thereafter until necropsy to determine the average daily weight gain.

2.6. Quantitative real-time PCR

PCV2: DNA-extraction on serum samples from all pigs collected on trial days 0, 7, 14, 21, 28, 35, and 42 was performed using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA). DNA-extracts were used for quantification of PCV2 genomic DNA copy numbers by real-time PCR as described previously (Opriessnig et al., 2003). In addition, DNA was extracted from a 10% fecal suspension obtained from all coinfected pigs (COINF, VAC-D-COINF, and VAC-M-COINF) on days 28 and 42 and used for PCV2 quantitative PCR. Fecal samples obtained from CONTROLS, PCV2-only, and PRRSV-only groups were not tested.
PRRSV: RNA-extraction was preformed on serum samples collected from all pigs on days 28, 35 and 42 using the QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). RNA-extracts were used for quantification of PRRSV genomic RNA copy numbers by real-time PCR as described previously (Opriessnig et al., 2006c).

2.7. Necropsy

All pigs were humanely euthanized by pentobarbital overdose and necropsied on day 42. Macroscopic lung lesions were estimated based on the amount of lung parenchyma affected by lesions and scores ranged from 0 to 100% (Halbur et al., 1995b). The

scoring system is based on the approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe, cranial part of the left cranial lobe, and the caudal part of the left cranial lobe contribute 10% each to the total lung volume, the accessory lobe contributes 5%, and the right and left caudal lobes contribute 27.5% each (Halbur et al., 1995b). Additionally, lymph node size was scored ranging from 0 (normal) to 3 (four times the normal size) and recorded (Opriessnig et al., 2006a). Lungs were insufflated with fixative as previously described (Halbur et al., 1995b). Sections of lymph nodes (tracheobronchial, mesenteric, mediastinal, superficial inguinal, and external iliac), tonsil, thymus, ileum, kidney, colon, spleen, and liver were collected at necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histological examination.

2.8. Histopathology

Microscopic lesions were evaluated by a pathologist blinded to the group designation of animal tissues. Lung sections were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 6 (severe diffuse) (Halbur et al., 1995b). Sections of heart, liver, kidney, ileum, and colon were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 (none) to 3 (severe). Lymphoid tissues including lymph nodes (tracheobronchial, mesenteric, mediastinal, superficial inguinal, and external iliac), tonsil, and spleen were evaluated for the presence of lymphoid depletion ranging from 0 (normal) to 3 (severe) and histiocytic inflammation and replacement of follicles ranging from 0 (normal) to 3 (severe) (Opriessnig et al., 2004b). The overall microscopic lymphoid lesions score which accounts for lymphoid depletion, histiocytic inflammation and PCV2-antigen present in lymphoid tissues was calculated as previously described (Opriessnig et al., 2004b) and ranged from 0 = normal to 9 = severe.

2.9. Immunohistochemistry

IHC for detection of PCV2-specific antigen was performed on selected formalin-fixed and paraffin-embedded sections of lymph nodes (tracheobronchial, mesenteric, mediastinal, superficial inguinal, and external iliac), tonsil, spleen, and thymus using a rabbit polyclonal antiserum (Sorden et al., 1999). PCV2-antigen scoring was done by a pathologist blinded to animal group designation. Scores ranged from 0 (no signal) to 3 (more than 50% of the lymphoid follicles contain cells with PCV2-antigen staining) (Opriessnig et al., 2004b). The mean group score was determined for each tissue and compared among groups.

IHC for detection of PRRSV-specific antigen was performed on lung sections as previously described (Halbur et al., 1995a).

2.10. Cytokine assays

ELISA-based assays were used to detect serum levels of selected cytokines to determine up or down regulation after challenge with PCV2, PRRSV, or both, compared to levels in control pigs (Petry et al., 2007). Cytokines detected by ELISA included interferon-gamma (IFNγ), interleukin-10 (IL-10) (Invitrogen/Biosource, Carlsbad, CA, USA), interferon-alpha (IFNα) (PBL Biomedical Laboratories, Piscataway, NJ, USA), interleukin-8 (IL-8), and interleukin-1beta (IL-1β) (R&D Systems, Minneapolis, MN, USA). All assays were performed according to manufacturers' specifications. Serum samples from 6 to 7 randomly selected pigs in each group collected on days 35 and 42 were tested for IFNγ, IL-10, IL-8, and IL-1β. Serum samples from all pigs in all groups collected on days 35 and 42 were tested for IFNα. The samples were run at 1:10, 1:4, and/or 1:2 dilutions of serum (assay dependent), respectively.

2.11. Statistical analysis

Summary statistics were calculated for all groups to assess the overall quality of the data, including normality. Continuous data were analyzed by using analysis of variance (ANOVA). If the *P*-value in the ANOVA was less than 0.05 the Tukey Kramer adjustment was used to determine which groups were different. In order to summarize and simplify the clinical observations, response feature analysis and a Chi-square test was used. Non-repeated measures of necropsy and histopathology data were assessed using non-parametric Kruskal–Wallis one-way ANOVA. If this non-parametric ANOVA test was significant

($P < 0.05$), then pairwise Wilcoxon tests were used to assess differences between groups. Differences in incidence were evaluated by using Fisher's exact test. The following sample analysis protocol was followed for the cytokine analysis: Each treatment group was randomly divided into two subgroups of 6–7 pigs each. All samples from the first group were assayed for IFNα, IFNγ, IL-1β, IL-8 and IL-10. If statistical differences between the first subgroups were detected, then the second subgroup of the serum samples was tested. For these repeat tests, statistics were affirmed for the second group, then both groups were combined, recalculated as one large group and data reported as the statistical analysis of all pigs.

3. Results

3.1. Clinical presentation

Mild respiratory disease characterized by sneezing and increased respiratory rates was observed after inoculation in the PRRSV-inoculated groups. There was a significant ($P < 0.001$) time by group interaction in rectal temperatures after PRRSV and PCV2 inoculation with the highest rectal temperatures in the PRRSV-only group (Fig. 1). The pigs and groups had a similar starting weight at the beginning of the trial. There were no significant differences in average weight gain until PCV2 and PRRSV inoculation on day 28. Between day 28 and 42, the average daily gain was significantly ($P < 0.001$) reduced in the PRRSV-only, COINF, VAC-D-COINF, and VAC-M-COINF pigs compared to CONTROLS and PCV2-only (data not shown). However, there was no difference in weight gain between the pigs inoculated with PRRSV regardless of vaccination- or PCV2-status.

3.2. Anti-PCV2-IgM antibody levels

The group anti-PCV2-IgM antibody levels are summarized in Fig. 2. All pigs were negative for anti-PCV2-IgM antibodies on days 0 and 7. On days 14, 21, and 28 the two vaccinated groups, VAC-M-COINF and VAC-D-COINF, had significantly ($P < 0.001$) higher amounts of anti-PCV2-IgM than all other groups. On days 35 and 42, PCV2-only and COINF pigs had significantly ($P < 0.001$) higher amounts of anti-PCV2-IgM compared to all other groups. Interestingly, the IgM response in the PCV2-only pigs was significantly ($P < 0.05$) higher compared to COINF on day 35 (7 days post-PCV2 and PRRSV-inoculation) (Fig. 2).

3.3. Anti-PCV2-IgG antibody levels

All pigs were negative for PCV2-specific anti-IgG antibodies at the beginning of the study. The group anti-PCV2-IgG antibody levels are summarized in Fig. 3. The vaccinated pigs seroconverted to PCV2 between 14 and 28 days post-vaccination, whereas the non-vaccinated pigs remained seronegative until after PCV2-challenge. VAC-M-COINF and VAC-D-COINF had significantly ($P < 0.001$) higher anti-PCV2-IgG antibody levels on days 35 and 42 post-vaccination compared to all other groups. PCV2-only had

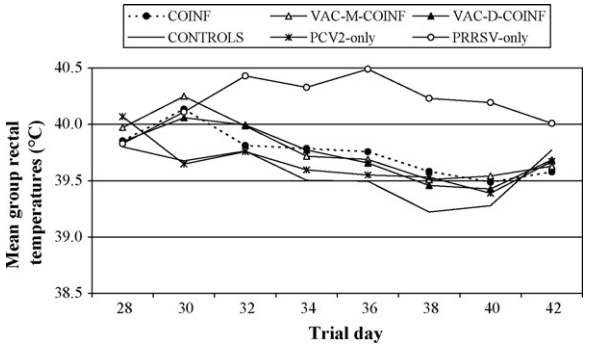

Fig. 1. Group mean rectal temperatures over time. Trial day 28 = day of PCV2 and PRRSV inoculation.

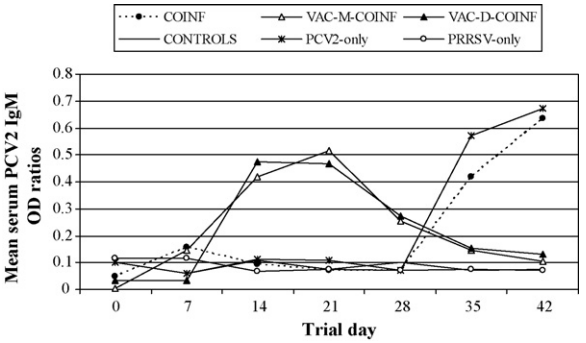

Fig. 2. Group mean optical density ratios and standard errors for anti-PCV2-IgM antibody response. Trial day 0 = day of PCV2 vaccination, trial day 28 = day of PCV2 challenge.

significantly ($P < 0.05$) higher anti-PCV2-IgG antibody levels on day 35 post-vaccination compared to PRRSV-only and CONTROLS but not to COINF. On day 42, both, PCV2-only and COINF groups, had significantly ($P < 0.05$) higher anti-PCV2-IgG antibody levels compared to PRRSV-only and CONTROLS.

3.4. Neutralizing anti-PCV2-antibodies

The neutralizing antibody response to PCV2 in all PCV2 inoculated groups (COINF, VAC-M-COINF, VAC-D-COINF, PCV2-only) is summarized in Fig. 4. On day 0, neutralizing antibodies were absent in all the pigs. On day 28, VAC-M-COINF and VAC-D-COINF had a neutralizing antibody response to the PCV2 vaccination 28 days earlier which was significantly ($P < 0.001$) different compared to the neutralizing antibody levels in COINF and PCV2-only pigs. On day 42, all pigs inoculated with PCV2 had anti-PCV2-neutralizing antibodies and the levels were significantly ($P < 0.001$) higher in VAC-M-COINF and VAC-D-COINF compared to COINF and PCV2-only pigs (Fig. 4).

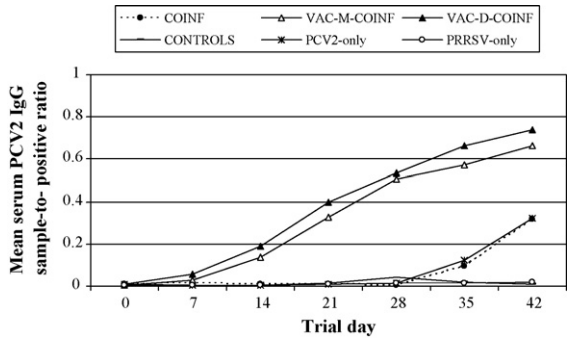

Fig. 3. Mean group anti-PCV2-IgG response over time. Trial day 0 = day of PCV2 vaccination, trial day 28 = day of PCV2 challenge. A sample-to-positive ratio equal or above 0.2 is considered positive.

3.5. Anti-PRRSV-IgG antibody levels

All pigs in all groups were negative for PRRSV-specific antibodies on day 28. On day 35, a portion of the pigs inoculated with PRRSV started to seroconvert (11/14 COINF, 9/12 VAC-M-COINF, 8/12 VAC-D-COINF, and 11/12 PRRSV-only), and on day 42, all PRRSV inoculated pigs had anti-PRRSV-IgG-antibodies above the ELISA-specific cut-off (data not shown).

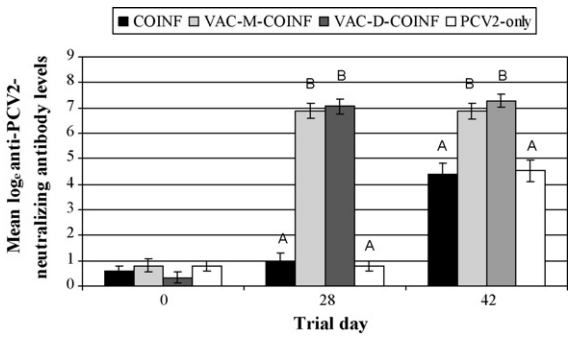

Fig. 4. Mean group anti-PCV2-neutralizing antibody response over time. Trial day 0 = day of PCV2 vaccination, trial day 28 = day of PCV2 challenge. Data are presented as $\log_e$ transformed group means ± S.E. If the bars are labeled with different letters (A, B) it indicates that the groups are significantly ($P < 0.05$) different from each other on that trial day.

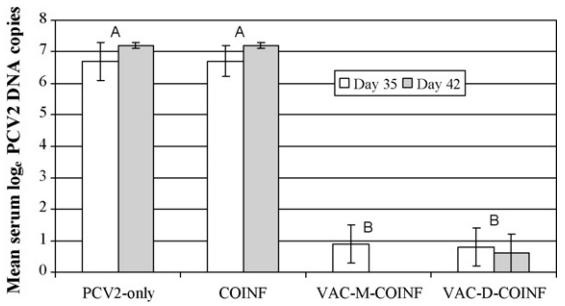

Fig. 5. Amount of PCV2 genomic copies per milliliter of serum 42 days post-vaccination which corresponds to 14 days post-PRRSV and PCV2-inoculation. Data are presented as $\log_e$ transformed group means ± S.E. If the bars are labeled with different letters (A, B) it indicates that the groups are significantly ($P < 0.05$) different from each other on that trial day.

3.6. Incidence and amount of PCV2 in sera and fecal samples

All pigs were negative for PCV2-DNA in serum until day 35 (7 days post-PCV2-inoculation). The results for days 35 and 42 are summarized in Fig. 5. On days 35 and 42, the amount of PCV2 DNA present in serum samples was significantly ($P < 0.05$) reduced in VAC-M-COINF and VAC-D-COINF compared to PCV2-only and COINF. The incidence of PCV2 DNA in serum was also significantly ($P < 0.05$) reduced in VAC-M-COINF (2/12; 0/12) and VAC-D-COINF (2/12; 1/12) compared to PCV2-only (11/12; 12/12) and COINF (14/14; 14/14) on both days. All coinfected pigs were negative for PCV2 DNA in feces on day 28. On day 42, VAC-M-COINF had a significantly ($P < 0.05$) reduced incidence of fecal PCR positive animals (4/12) compared to COINF (13/14) but not compared to VAC-D-COINF (9/12). In addition the amount of PCV2 DNA in feces was significantly ($P < 0.05$) reduced in VAC-M-COINF (group $\log_e$ mean ± S.E.; 1.18 ± 0.51) and VAC-D-COINF (2.75 ± 0.48) compared to COINF (5.83 ± 0.23).

3.7. Incidence and amount of PRRSV in sera

All pigs were negative for PRRSV-RNA in serum until day 35 (7 days post-PRRSV-inoculation). The incidence of PRRSV-RNA in serum was not different between PRRSV-inoculated groups on day 35 and on day 42. Similarly, the $\log_e$ transformed mean amount of PRRSV RNA genomic copies per ml serum on day 35 was not different between PRRSV-inoculated groups (5.3 ± 0.1 in PRRSV-only, 5.1 ± 0.1 in VAC-M-COINF, 5.3 ± 0.1 in VAC-D-COINF, and 4.8 ± 0.4 in COINF). On day 42, the $\log_{10}$ transformed group mean in the COINF pigs (4.4 ± 0.1) was significantly ($P < 0.05$) higher compared to the mean in the PRRSV-only pigs (3.4 ± 0.3) whereas the means in the VAC-M-COINF (4.1 ± 0.2) and VAC-D-COINF (4.2 ± 0.3) were not different from either of the two former groups.

3.8. Macroscopic lesions

Macroscopic lesions were characterized by mottled-tan lungs of varying degrees of severity and mild-to-moderate enlargement of lymph nodes. As expected, COINF pigs had the highest mean group gross lung lesion scores (Fig. 6). Both PCV2-vaccinated groups had reduced lung lesion scores; however, only the VAC-M-COINF pigs had a significant ($P < 0.05$) reduction of the percentage of the lung surface affected by lesions compared to COINF (Fig. 6).

3.9. Microscopic lesions and incidence of PCV2 and PRRSV antigen in tissues

Lung tissues had focal-to-diffuse, mild-to-severe, lymphohistiocytic interstitial pneumonia characterized by type 2 pneumocyte hypertrophy and hyperplasia and mild multifocal necrotizing bronchiolitis with fibroplasia. The mean microscopic lung lesion

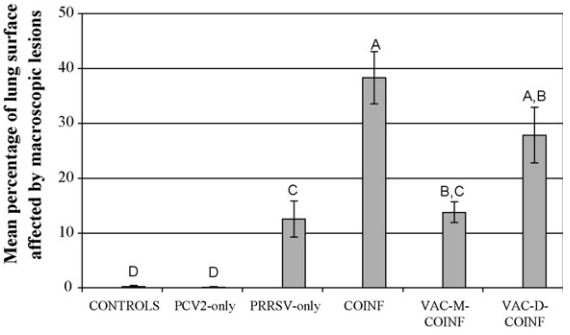

Fig. 6. Gross lung scores (percentage of lung affected by lesions) 42 days post-vaccination which corresponds to 14 days post-PRRSV and PCV2-inoculation. Data are presented as group means ± S.E. If the bars are labeled with different letters (A, B, C, D) it indicates that the groups are significantly ($P < 0.05$) different from each other.

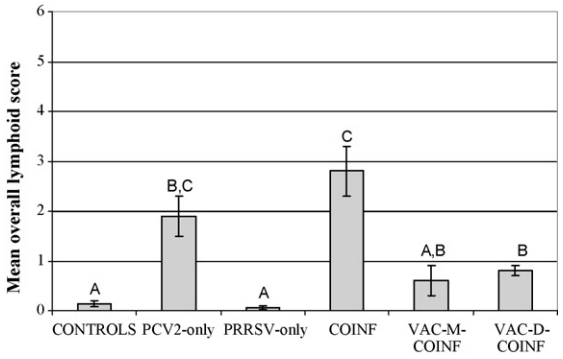

Fig. 7. Mean group overall lymphoid lesions (lymphoid depletion, histiocytic replacement, amount of PCV2-antigen as determined by immunohistochemistry) 42 days post-vaccination which corresponds to 14 days post-PRRSV and PCV2-inoculation. Data are presented as group means ± S.E. If the bars are labeled with different letters (A, B) it indicates that the groups are significantly ($P < 0.05$) different from each other.

scores were lowest in the CONTROLS (0.5 ± 0.1) and in the PCV2-only pigs (1.1 ± 0.3). PRRSV-only pigs had significantly ($P < 0.05$) increased mean group score (2.7 ± 0.4) and the highest scores were seen in the coinfected groups. The mean group scores were significantly ($P < 0.05$) higher in both COINF (4.3 ± 0.3) and VAC-D-COINF (4.3 ± 0.3) (4.3 ± 0.3) pigs, whereas the mean VAC-M-COINF score (3.8 ± 0.3) was neither significantly ($P = 0.551$) different from PRRSV-only nor from VAC-D-COINF. The incidence of PRRSV antigen in lung sections as determined by IHC stains was 10/14 for COINF, 7/12 for VAC-M-COINF, 8/12 for VAC-D-COINF, and 4/12 for PRRSV-only. The PRRSV antigen incidence was significantly ($P = 0.04$) higher in COINF pigs compared to PRRSV-only pigs. The incidence of PCV2-antigen in lung sections was 4/14 in COINF, 0/12 for VAC-M-COINF, 0/12 for VAC-D-COINF, and 3/12 for PCV2-only.

Lymphoid lesions were characterized by mild-to-moderate depletion of follicles and mild granulomatous lymphadenitis in individual PCV2 challenged pigs and there was low-to-moderate amounts of PCV2-antigen associated with the lesions. The overall lymphoid score was significantly ($P < 0.001$) lower in both vaccinated groups (VAC-M-COINF, VAC-D-COINF) compared to COINF pigs (Fig. 7).

3.10. Cytokine response

On day 35 (7 days post-PCV2 and PRRSV-inoculation) there was an increase in serum IFNγ levels in COINF, VAC-D-COINF, and VAC-M-COINF groups compared to the other groups; however, this was not significant (Table 2). By day 42 serum IFNγ had returned to control levels. Mean group serum IFNα levels were significantly ($P < 0.05$) increased in COINF pigs compared to CONTROLS and VAC-D-COINF pigs on day 35 (Table 2); however, they were below detection limits and not different between groups on day 42 (data not shown). The IL-10 and IL-1β serum levels were below detection limits for all pigs in all groups.

4. Discussion

Under the conditions of this study, PCV2-vaccination 28 days prior to coinfection with PCV2 and

Table 2
Mean group interferon-gamma (IFNγ) and interleukin-8 (IL-8) on day 35 and 42 ($n = 6$–7 pigs) and mean interferon-alpha (IFNα) concentrations on day 35 ($n = 12$–14 pigs)

Group	IFNγ		IL-8		IFNα
	Day 35	Day 42	Day 35	Day 42	Day 35
CONTROLS	29.1 ± 8.7	20.7 ± 3.5	96.6 ± 37.4	109.6 ± 30.7	32.0 ± 10.8^{A}
PCV2-only	26.3 ± 5.7	19.1 ± 3.1	55.1 ± 6.6	109.8 ± 24.6	$60.1 \pm 10.8^{A,B}$
PRRSV-only	21.6 ± 5.5	23.3 ± 4.0	70.4 ± 21.1	81.0 ± 23.5	$58.1 \pm 10.8^{A,B}$
COINF	43.2 ± 16.4	17.9 ± 1.0	64.9 ± 15.4	54.7 ± 11.4	80.1 ± 10.0^{B}
VAC-M-COINF	36.3 ± 18.6	29.6 ± 7.3	69.7 ± 8.3	62.9 ± 7.4	$58.8 \pm 10.8^{A,B}$
VAC-D-COINF	40.9 ± 18.5	20.1 ± 4.3	64.3 ± 9.3	84.7 ± 13.1	49.4 ± 10.8^{A}

Significantly different ($P < 0.05$) group means within each column are indicated by different superscripts (A, B). The data are presented as group mean concentration in pg/ml ± standard error.

PRRSV induced a strong IgM, IgG and neutralizing antibody response and vaccinated pigs had significantly decreased amounts and incidence of PCV2-DNA in sera, feces, and tissues compared to non-vaccinated coinfected pigs. Limited changes were observed in serum IFNγ, IFNα and IL-8 levels post-challenge and there was no correlation of serum cytokine levels with protection against PCV2 challenge.

In experimental PCV2-PRRSV coinfection models, PRRSV is known to enhance PCV2 replication and associated lesions; however, the outcome of PRRSV infection was found not to be influenced by PCV2 infection (Allan et al., 2000a). The quantities and distribution of PRRSV antigen in pigs singularly inoculated with PRRSV and in pigs coinfected with PRRSV and PCV2 were found to be similar (Allan et al., 2000a) and these findings were confirmed in the current study. In contrast, Rovira et al. (2002) found that PRRSV RNA was present for a longer period of time in serum in pigs dually inoculated with PRRSV and PCV2 compared to single-PRRSV-inoculated pigs; however, that difference was demonstrated at 28 days post-PRRSV-PCV2 inoculation and our study was terminated at 14 days post-inoculation.

In the current study and in contrast to what has been reported in other experimental PCV2-PRRSV-coinfection models (Allan et al., 2000a,b; Rovira et al., 2002), the severity of PCV2-induced lymphoid lesions (Fig. 7) and the amount of PCV2 DNA in sera (Fig. 5) was not significantly different in coinfected pigs compared to pigs singularly infected with PCV2. Compared to the PRRSV-only pigs, the coinfected pigs had significantly ($P < 0.05$) reduced rectal temperatures after PRRSV inoculation (Fig. 1) which is in contrast to the study by Rovira et al. (2002) where an increase in rectal temperatures was observed in coinfected pigs.

The PCV2 isolate and the PRRSV isolate used in this experiment are considered typical of current U.S. field isolates and both have been extensively characterized *in vitro* and in growing pig models (Halbur et al., 1995b, 1996; Opriessnig et al., 2003, 2004a,b, 2006b,c,d). Differences in virulence among PCV2 isolates (Opriessnig et al., 2006d) and among PRRSV (Halbur et al., 1995b, 1996) isolates have been reported. The PCV2 isolate used in this study is considered of moderate virulence (Opriessnig et al., 2006d). Evidence of differences in host susceptibility to PCV2 (Opriessnig et al., 2006a) and PRRSV (Halbur et al., 1998; Vincent et al., 2005) infection has also been reported. It is possible that other isolates of PRRSV and PCV2 and the use of different breeds of pigs in the model may influence the outcome of the coinfection. Similarly, the timing and sequence of the coinfection may affect the outcome.

In experimental models, PCV2 replication and associated microscopic lesions have been shown to be upregulated by immunostimulation as induced by routine vaccination against other swine pathogens (Allan et al., 2000b, 2001) and this is thought to be adjuvant related (Hoogland et al., 2006). A proposed mechanism of the enhancement of PCV2 replication is thought to be due to stimulation of the monocyte/macrophage lineage and other cells of the immune system (Allan et al., 2000a; Krakowka et al., 2001). Whereas the efficacy of PCV2 vaccines has been proven in the PCV2 challenge model (Fenaux et al., 2004; Opriessnig et al., 2008), no information was available on its efficacy in the experimental coinfection model. The concurrent presence of adjuvants (from the PCV2 vaccine) and PRRSV could potentially result in enhancement of PCV2 replication rather than controlling it. In this study, we showed for the first time that a killed, adjuvanted PCV2 vaccine is effective in controlling PCV2 in an experimental PCV2-PRRSV coinfection model that mimics the field situation and confirmed that evidence for vaccine-induced enhancement of disease was lacking.

The PCV2-challenge in this study was done by both intramuscular and intranasal routes which is in contrast to other groups who used the intranasal route only (Allan et al., 2003; Rovira et al., 2002). The combination of intranasal and intramuscular routes of inoculation have been used previously by our group (Opriessnig et al., 2006a,b) and was found to be successful in obtaining a uniform PCV2 infection level in the pigs. We chose to do both because when using only the intranasal route the likelihood increases that not all the pigs inhale an equal amount of inoculum due to sneezing and/or labored breathing at the time of inoculation.

It would have been ideal to include a group of vaccinated pigs that were singularly inoculated with PRRSV or PCV2 but this was not possible due to limitations with finding large numbers of PCV2-naive

pigs, space limitations, and cost issues. Since the incidence of clinical manifestation of disease and PCV2-associated lesions in PCV2-inoculated pigs has a relatively low prevalence, it was important to maximize the number of pigs in the other treatment groups in order to be able to demonstrate significance in observed differences between groups if present.

PCV2 vaccination alone was not sufficient to entirely eliminate the detrimental effect of PCV2 in coinfected pigs. However, the effect of PCV2 vaccination on incidence and amount of PCV2 was dramatic with significant decreases in incidence and amount of PCV2 in lung sections, lymphoid sections, and sera. Seven days post-inoculation (day 35), the incidence of PCV2 DNA positive animals was 16.7% (4/24) for vaccinated pigs, 91.7% (11/12) for PCV2-only, and 100% for unvaccinated coinfected pigs. Fourteen days post-inoculation (day 42), the incidence of PCV2 DNA positive animals was 4.2% (1/24) for vaccinated pigs, and 100% for both the unvaccinated and PCV2 singularly infected (12/12) and the unvaccinated coinfected (14/14) pigs.

The use of the same needle to intramuscularly vaccinate a large number of pigs is known to be a risk factor for spreading diseases within a group. The main advantage of the intradermal administration is increased safety due to elimination of the needle (Laurent et al., 2007). Intradermal vaccination is also likely to lessen the risk of disease transmission within the group related to the vaccination process. There were no significant differences in the parameters measured between the two vaccination strategies indicating that both were similarly effective in reducing the effect of PCV2 in vaccinated pigs.

The group average IFNγ, IL-8, IL-10 and IL-1β serum levels were not different among the pigs in the different treatment groups in this study. Darwich et al. (2003) found altered cytokine mRNA expression profiles in lymphoid tissues of pigs naturally PCV2 exposed and clinically PCVAD affected with dysregulation of IL-10 and IFNγ. In another group of clinically affected, naturally exposed PCVAD pigs, intracellular IL-10 and INFγ levels were increased; however, similar to the current study this was not significant (Sipos et al., 2004). In the current study, IFNα levels were found to be significantly upregulated by concurrent PRRSV and PCV2 infection as compared to CONTROLS and VAC-D-COINF 7 days post-inoculation (day 35). IFNα was previously found to be induced by PCV2 in swine alveolar macrophages (Chang et al., 2005). When swine alveolar macrophages were concurrently infected with PCV2 and PRRSV, PRRSV infection and PRRSV-associated cytopathic effect were reduced. The authors connected these findings with IFNα induction by PCV2 (Chang et al., 2005). This may also explain why PRRSV was not upregulated by concurrent PCV2 infection *in vivo* as demonstrated in the current study.

In order to more closely mimic what occurs in the field (Pallarés et al., 2002; Harms et al., 2002), the pigs in this study were coinfected with PRRSV and PCV2. The economic impact of PCVAD is most important in growing pigs and concurrent PCV2 and PRRSV infection is one of the most frequent combinations found in the field (Dorr et al., 2007; Pallarés et al., 2002). PRRSV is widely spread in the global swine population and vaccination strategies for PRRSV have yielded mixed results. In pigs naturally coinfected with PCV2 and PRRSV, the use of a modified live PRRSV vaccine in the piglets and dams significantly reduced mortality as compared to non-vaccinated piglets from non-vaccinated sows (Kritas et al., 2007). However, in the colostrum-deprived pig model, a modified live PRRSV vaccine increased the severity of microscopic lesions and PCV2 antigen lesions in pigs that received PCV2 inoculum 1 week prior to vaccination compared to pigs that were not vaccinated (Allan et al., 2007). Moreover, the efficacy of a modified-live PRRSV vaccine was reduced when administered to pigs previously inoculated with PCV2 (Opriessnig et al., 2006c).

In summary, PCV2 vaccination was effective at inducing a neutralizing antibody response and significantly reducing PCV2-associated lesions and PCV2 viremia in pigs coinfected with PCV2 and PRRSV. Differences between intradermal and intramuscular routes of vaccine administration were not observed.

Acknowledgments

We wish to thank Paul Thomas for assistance with animal work and Dr. Igor Morozov from Fort Dodge Animal Health, Inc. for providing the inocula.

References

Allan, G., McNeilly, F., Meehan, B., McNair, I., Ellis, J., Krakowka, S., Fossum, C., Wattrang, E., Wallgren, P., Adair, B., 2003. Reproduction of postweaning multisystemic wasting syndrome in pigs experimentally inoculated with a Swedish porcine circovirus isolate. J. Vet. Diagn. Invest. 15, 553–560.

Allan, G.M., Caprioli, A., McNair, I., Lagan-Tregaskis, P., Ellis, J., Krakowka, S., McKillen, J., Ostanello, F., McNeilly, F., 2007. Porcine circovirus 2 replication in colostrum-deprived piglets following experimental infection and immune stimulation using a modified live vaccine against porcine respiratory and reproductive syndrome virus. Zoonoses Public Health 54, 214–222.

Allan, G.M., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I., Kennedy, S., 2000a. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Arch. Virol. 145, 2421–2429.

Allan, G.M., McNeilly, F., Kennedy, S., Meehan, B., Ellis, J., Krakowka, S., 2000b. Immunostimulation, PCV-2 and PMWS. Vet. Rec. 147, 170–171.

Allan, G.M., McNeilly, F., McNair, I., O'Connor, M., Meehan, B., Gilpin, D., Ellis, J., Townsend, H., Lasagna, C., Boriosi, G., Krakowka, S., 2001. Neonatal vaccination for *Mycoplasma hyopneumoniae* and post-weaning multisystemic wasting syndrome: a field trial. Pig. J. 48, 34–41.

Chang, H.W., Jeng, C.R., Liu, J.J., Lin, T.L., Chang, C.C., Chia, M.Y., Tsai, Y.C., Pang, V.F., 2005. Reduction of porcine reproductive and respiratory syndrome virus (PRRSV) infection in swine alveolar macrophages by porcine circovirus 2 (PCV2)-induced interferon-alpha. Vet. Microbiol. 108, 167–177.

Darwich, L., Pié, S., Rovira, A., Segalés, J., Domingo, M., Oswald, I.P., Mateu, E., 2003. Cytokine mRNA expression profiles in lymphoid tissues of pigs naturally affected by postweaning multisystemic wasting syndrome. J. Gen. Virol. 84, 2117–2125.

Dorr, P.M., Baker, R.B., Almond, G.W., Wayne, S.R., Gebreyes, W.A., 2007. Epidemiologic assessment of porcine circovirus type 2 coinfection with other pathogens in swine. J. Am. Vet. Med. Assoc. 230, 244–250.

Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.J., 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. 38, 2494–2503.

Fenaux, M., Halbur, P.G., Haqshenas, G., Royer, R., Thomas, P., Nawagitgul, P., Gill, M., Toth, T.E., Meng, X.J., 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J. Virol. 76, 541–551.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J. Virol. 78, 6297–6303.

Halbur, P.G., Miller, L.D., Paul, P.S., Meng, X.J., Huffman, E.L., Andrews, J.J., 1995a. Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrum-deprived pigs. Vet. Pathol. 32, 200–204.

Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1995b. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. 32, 648–660.

Halbur, P.G., Paul, P.S., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1996. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. J. Vet. Diagn. Invest. 8, 11–20.

Halbur, P.G., Rothschild, M.F., Thacker, B.J., Meng, X.J., Paul, P.S., Bruna, J.D., 1998. Differences in susceptibility of Duroc, Hampshire, and Meishan pigs to infection with a high virulence strain (VR2385) of porcine reproductive and respiratory syndrome virus (PRRSV). J. Anim. Breed. Genet. 115, 181–189.

Harding, J.C.S., Clark, E.G., 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health Prod. 5, 201–203.

Harms, P.A., Halbur, P.G., Sorden, S.D., 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. J. Swine Health Prod. 10, 27–30.

Harms, P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, I., Paul, P.S., 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. 38, 528–539.

Hoogland, M.J., Opriessnig, T., Halbur, P.G., 2006. Effects of adjuvants on porcine circovirus type 2-associated lesions. J. Swine Health Prod. 14, 133–139.

Krakowka, S., Ellis, J.A., McNeilly, F., Ringler, S., Rings, D.M., Allan, G., 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol. 38, 31–42.

Kritas, S.K., Alexopoulos, C., Kyriakis, C.S., Tzika, E., Kyriakis, S.C., 2007. Performance of fattening pigs in a farm infected with both porcine reproductive and respiratory syndrome (PRRS) virus and porcine circovirus type 2 following sow and piglet vaccination with an attenuated PRRS vaccine. J. Vet. Med. A Physiol. Pathol. Clin. Med. 54, 287–291.

Laurent, P.E., Bonnet, S., Alchas, P., Regolini, P., Miszta, J.A., Pettis, R., Harvey, N.G., 2007. Evaluation of the clinical performance of a new intradermal vaccine administration technique and associated delivery system. Vaccine 25, 8833–8842.

Nawagitgul, P., Harms, P.A., Morozov, I., Thacker, B.J., Sorden, S.D., Lekcharoensuk, C., Paul, P.S., 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clin. Diagn. Lab. Immunol. 9, 33–40.

Opriessnig, T., Fenaux, M., Thomas, P., Hoogland, M.J., Rothschild, M.F., Meng, X.J., Halbur, P.G., 2006a. Evidence of breed-

dependent differences in susceptibility to porcine circovirus type-2-associated disease and lesions. Vet. Pathol. 43, 281–293.

Opriessnig, T., Fenaux, M., Yu, S., Evans, R.B., Cavanaugh, D., Gallup, J.M., Pallares, F.J., Thacker, E.L., Lager, K.M., Meng, X.J., Halbur, P.G., 2004a. Effect of porcine parvovirus vaccination on the development of PMWS in segregated early weaned pigs coinfected with type 2 porcine circovirus and porcine parvovirus. Vet. Microbiol. 98, 209–220.

Opriessnig, T., Halbur, P.G., Yu, S., Thacker, E.L., Fenaux, M., Meng, X.J., 2006b. Effects of the timing of the administration of *Mycoplasma hyopneumoniae* bacterin on the development of lesions associated with porcine circovirus type 2. Vet. Rec. 158, 149–154.

Opriessnig, T., McKeown, N.E., Harmon, K.L., Meng, X.J., Halbur, P.G., 2006c. Porcine circovirus type 2 infection decreases the efficacy of a modified live porcine reproductive and respiratory syndrome virus vaccine. Clin. Vac. Immunol. 13, 923–929.

Opriessnig, T., McKeown, N.E., Zhou, E.M., Meng, X.J., Halbur, P.G., 2006d. Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. J. Gen. Virol. 87, 2923–2932.

Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J. Vet. Diagn. Invest. 19, 591–615.

Opriessnig, T., Patterson, A.R., Elsener, J., Meng, X.J., Halbur, P.G., 2008. Influence of porcine circovirus type 2 (PCV2) maternal antibodies on efficacy of PCV2 vaccination to protect pigs from experimental infection with PCV2. Clin. Vac. Immunol. 2007 (Available on PubMed).

Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.J., Halbur, P.G., 2004b. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. Vet. Pathol. 41, 624–640.

Opriessnig, T., Yu, S., Gallup, J.M., Evans, R.B., Fenaux, M., Pallares, F., Thacker, E.L., Brockus, C.W., Ackermann, M.R., Thomas, P., Meng, X.J., Halbur, P.G., 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. Vet. Pathol. 40, 521–529.

Pallarés, F.J., Halbur, P.G., Opriessnig, T., Sorden, S.D., Villar, D., Janke, B.H., Yaeger, M.J., Larson, D.J., Schwartz, K.J., Yoon, K.J., Hoffman, L.J., 2002. Porcine circovirus type 2 (PCV-2) coinfections in US field cases of postweaning multisystemic wasting syndrome (PMWS). J. Vet. Diagn. Invest. 14, 515–519.

Petry, D.B., Lunney, J., Boyd, P., Kuhar, D., Blankenship, E., Johnson, R.K., 2007. Differential immunity in pigs with high and low responses to porcine reproductive and respiratory syndrome virus infection. J. Anim. Sci. 85, 2075–2092.

Pogranichnyy, R.M., Yoon, K.J., Harms, P.A., Swenson, S.L., Zimmerman, J.J., Sorden, S.D., 2000. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunol. 13, 143–153.

Rovira, A., Balasch, M., Segalés, J., Garcia, L., Plana-Dúran, J., Rosell, C., Ellerbrok, H., Mankertz, A., Domingo, M., 2002. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. J. Virol. 76, 3232–3239.

Sipos, W., Duvigneau, J.C., Willheim, M., Schilcher, F., Hartl, R.T., Hofbauer, G., Exel, B., Pietschmann, P., Schmoll, F., 2004. Systemic cytokine profile in feeder pigs suffering from natural postweaning multisystemic wasting syndrome (PMWS) as determined by semiquantitative RT-PCR and flow cytometric intracellular cytokine detection. Vet. Immunol. Immunopathol. 99, 63–71.

Sorden, S.D., Harms, P.A., Nawagitgul, P., Cavanaugh, D., Paul, P.S., 1999. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. J. Vet. Diagn. Invest. 11, 528–530.

Vincent, A.L., Thacker, B.J., Halbur, P.G., Rothschild, M.F., Thacker, E.L., 2005. In vitro susceptibility of macrophages to porcine reproductive and respiratory syndrome virus varies between genetically diverse lines of pigs. Viral Immunol. 18, 506–512.